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Enzymic Synthesis of an Aromatic Ring from Acetate Units

Partial Purification and Some Properties of Flavanone Synthase from Cell-Suspension Cultures of *Petroselinum hortense*

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Flavanone synthase was isolated and purified about 300-fold from fermenter-grown, light-induced cell suspension cultures of *Petroselinum hortense*. The enzyme catalyzed the formation of the flavanone naringenin from *p*-coumaroyl-CoA and malonyl-CoA. Trapping experiments with an enzyme preparation, which was free of chalcone isomerase activity, revealed that in fact the flavanone and not the isomeric chalcone was the immediate product of the synthase reaction. Thus the enzyme is not a chalcone synthase as previously assumed.

No cofactors were required for flavanone synthase activity. The enzyme was strongly inhibited by the two reaction products naringenin and CoASH, by the antibiotic cerulenin, by acetyl-CoA, and by several compounds reacting with sulfhydryl groups. Optimal enzyme activity was found at pH 8.0, at 30 °C, and at an ionic strength of 0.1–0.3 M potassium phosphate. EDTA, Mg^{2+}, Ca^{2+}, or Fe^{2+} at concentrations of about 0.7 µM did not affect the enzyme activity. Apparent molecular weights of approx. 120000, 50000, and 70000, respectively, were determined for flavanone synthase and two metabolically related enzymes, chalcone isomerase and malonyl-CoA : flavonoid glycoside malonyl transferase.

The partially purified flavanone synthase efficiently catalyzed the formation of malonyl pantetheine from malonyl-CoA and pantetheine. This malonyl transferase activity, and a general similarity with the condensation steps involved in the mechanisms of fatty acid and 6-methyl-salicylic acid synthesis from "acetate units", are the basis for a hypothetical scheme which is proposed for the sequence of reactions catalyzed by the multifunctional flavanone synthase.

The first enzyme of the flavonoid pathway has long been of special theoretical interest. In spite of a recent successful attempt to demonstrate the synthesis of the flavanone naringenin from *p*-coumaroyl-CoA and malonyl-CoA in a cell-free system [1], the question still remained open whether in fact the flavanone or the isomeric chalcone (*cf.* Fig. 6) was the more immediate product of the condensation reaction. In particular, a complete separation of the synthase activity from all contaminating chalcone isomerase activity was a necessary prerequisite before this question could be answered by experimental results at an enzymic level.

A second aspect of theoretical interest is the formation of the aromatic ring A of the flavonoid product from three molecules of malonyl-CoA during the condensation reaction. This type of reaction has long been postulated for the biosynthesis of flavonoids [2] as an example for the application of the "acetate rule" to the synthesis of various aromatic ring structures [3, 4]. The formation of naringenin from *p*-coumaroyl-CoA as the primer molecule and malonyl-CoA as the origin of the acetate units might serve as an especially suitable model system for studying the mechanism of such a condensation reaction. The substitution patterns of the malonate-derived ring A and the *p*-coumarate-derived ring B of naringenin suggest that catalytic activities other than those related to the condensation steps are not involved in the overall reaction of the synthase.

Previous studies of the flavanone synthase from cell suspension cultures of *Petroselinum hortense* have been primarily concerned with the identification of the product, the localization of the radioactivity

Enzymes. Chalcone isomerase (EC 5.5.1.6); malonyl-CoA : flavonoid glycoside malonyl transferase (EC 2.3.1.–).

incorporated from ^{14}C-labelled malonyl-CoA [1], changes in the enzyme activity after irradiation of the cells [5], and initial attempts to purify the enzyme [6]. In this communication we report an extensive purification and some properties of the flavanone synthase.

Until recently, a quantitative estimation of the enzyme activity had been complicated under certain conditions by the formation of a second product whose chemical structure had been unknown [6]. This compound has now been identified as bis-noryangonin (4-hydroxy-6-[4-hydroxystyryl]-2-pyrone, see Fig. 6), and the conditions of its formation have been studied [7]. The sum of both products, naringenin and bis-noryangonin, remained approximately constant under most conditions of the enzyme assay and will therefore frequently be used in the present communication as a measure of the synthase activity.

MATERIALS AND METHODS

[2-^{14}C]Malonyl-CoA (20.5 mCi/mmol) and [2-^{14}C]-acetyl-CoA (59 mCi/mmol) were obtained from New England Nuclear (Boston, Mass.); ATP, CoASH, and *N*-ethylmaleimide from Boehringer, Mannheim; iodoacetamide, *p*-chloromercuribenzoate, avidin, and unlabelled malonyl-CoA from Serva, Heidelberg. Pantetheine was prepared immediately before use by reduction of pantethine (Sigma, St. Louis) with a 4-fold molar concentration of mercaptoethanol for 15 min at 30 °C [8]. *p*-Coumaroyl-CoA was synthesized as described previously [9]. Naringenin and 4,2′,4′,6′-tetrahydroxychalcone were from our laboratory collection. Cerulenin was a gift from S. Omura, Tokio.

Buffers

The two buffers mostly used were (A) 100 mM and (B) 10 mM potassium phosphate, pH 8.0, each containing 1.4 mM mercaptoethanol.

Cell Suspension Cultures

Cell suspension cultures of parsley (*Petroselinum hortense* Hoffm.) were propagated at 26 °C in a fermenter containing 10 l B5 medium [10] which was modified by using 50 µM $FeSO_4$ – EDTA as a source of iron. The fermenter was agitated at 200 rev./min, aerated at 2 l/min, and inoculated with 3 seven-day-old rotary shaking cultures, each containing about 80 g of cells (fresh weight) in 400 ml [11]. Growth of the culture was measured by monitoring the conductivity of the medium as in previous experiments [12, 13]. After about 7–8 days in the dark [13], the culture was irradiated for 24 h with 16 Osram-L 20 W/73 lamps through the glass vessel. The cells (approximately 2.5 kg fresh weight) were then harvested on a porous glass filter, frozen with liquid nitrogen, and stored at – 18 °C. No significant loss of flavanone synthase activity was observed under these conditions within several months.

Purification of the Enzyme

All of the following steps were carried out at 4 °C. 300 g of frozen cells were thawed and homogenized for 30 min in a mortar with 150 ml of buffer A. The homogenate was cleared by centrifugation for 15 min at 15000 × *g*, and the supernatant was stirred for 20 min with 30 g Dowex 1X2 (phosphate form) equilibrated with 20 mM potassium phosphate (pH 7.5). The resin was then removed by filtration through glass wool, and 525 ml of a saturated, aqueous solution of $(NH_4)_2SO_4$ were slowly added within 45 min to 350 ml of the protein extract. The mixture was stirred for an additional 15 min, and the precipitate was removed by centrifugation for 15 min at 15000 × *g*. A second precipitate was obtained from the supernatant by adding 875 ml of the saturated $(NH_4)_2SO_4$ solution in the same manner. This precipitate was dissolved in 12 ml of buffer A, and the solution was passed through a Sephadex G-25 column (2 × 60 cm) using buffer B. The flavanone synthase activity was eluted in a total volume of about 35 ml (8 mg protein/ml) and stored at – 18 °C until further use (solution S). After an initial loss of about 30% upon freezing, no further decrease in the enzyme activity occurred under these conditions at least within several weeks.

Solution S was applied to a DEAE-cellulose column (3.2 × 13 cm) which had been equilibrated with buffer B. Proteins were eluted at a flow rate of 80 ml/h first with 100 ml of buffer B, and then with a linear gradient of approx. 1.2 l of this buffer, increasing in molarity from 10 to 200 mM potassium phosphate. Fractions of 14.8 ml were collected, and the potassium phosphate concentration was determined by measuring the conductivity. Those fractions (about 200 ml) containing most of the flavanone synthase activity (see Fig. 1) were concentrated to about 10 ml by ultrafiltration. This solution was passed through the same Sephadex G-25 column as described above, using buffer B. The resulting protein solution (27 ml) was applied to a hydroxyapatite column (1.9 × 7 cm) which had been equilibrated with buffer B at pH 7.8. The bulk of the protein was eluted with buffer A at pH 7.8 until the eluate was free of protein. The flavanone synthase was then eluted at a flow rate of 50 ml/h with about 80 ml 0.2 M potassium phosphate buffer, pH 7.8, containing 1.4 mM mercaptoethanol. The

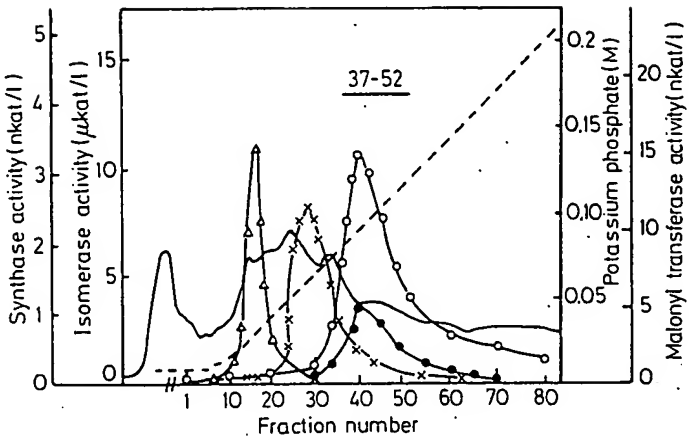

Fig. 1. *Separation of flavanone synthase (○), chalcone isomerase (△) and malonyl-CoA:flavonoid glycoside malonyl transferase (×) on a DEAE-cellulose column.* Enzyme activities were determined using the standard assays as described under Materials and Methods. For the calculation of flavanone synthase activity, the sum of both products, naringenin (○; 75%) and bis-noryangonin (●; 25%), was used. (----) Potassium phosphate gradient; (——) absorbance at 280 nm (arbitrary units). The bar indicates the fractions collected for further purification of the synthase

enzyme solution was concentrated by ultrafiltration to about 30 ml and immediately used for further experiments (solution H).

Enzyme Assays

The standard assay mixture for measuring flavanone synthase activity contained 1 – 100 µg protein (depending on the degree of purity), 1 nmol *p*-coumaroyl-CoA, 2.44 nmol [2-^{14}C]malonyl-CoA (1.1×10^5 dis./min), 0.14 µmol mercaptoethanol, and 10 µmol potassium phosphate, pH 8.0, in a total volume of 110 µl. Aliquots of acidic aqueous stock solutions of the CoA esters were adjusted with 0.1 M KOH to pH 8.0 before addition to the assay mixture. The incubation was carried out at 30 °C for 20 min and then stopped by adding a solution of 30 µg naringenin in 40 µl methanol – acetic acid (1:1, by vol.). The mixture was evaporated on Schleicher & Schüll No. 2043b paper and chromatographed in 15% ethanol. Naringenin and, if present, bis-noryangonin were detected under ultraviolet light of 350 nm or with a radiochromatogram scanner LB 280 (Berthold, Wildbad). The spots corresponding to these two products [7] were cut out, and the radioactivity was measured by scintillation spectrometry using a solution of 5 g PPO in 1 l toluene (50% counting efficiency).

Chalcone isomerase [14] and malonyl-CoA:flavonoid glycoside malonyl transferase [15] activities were determined according to previously published procedures.

The malonyl transfer reaction of the flavanone synthase was measured by incubating a mixture of 2.44 nmol [2-^{14}C]malonyl-CoA, 50 nmol pantetheine, 0.14 µmol mercaptoethanol, and 10 µmol potassium phosphate, pH 8.0, in 100 µl of solution H (see above) at 30 °C for 20 min. The reaction was stopped by adding 20 µl acetic acid. The mixture was evaporated on Schleicher & Schüll No. 2043b paper and chromatographed in isobutyric acid – ammonia (25%) – water (66:1:30, by vol.). The radioactivity associated with the spots corresponding to malonyl pantetheine ($R_F = 0.61$) and malonyl-CoA ($R_F = 0.3$) was determined as described above for naringenin and bis-noryangonin.

Flavanone Synthase Assay in the Presence of Chalcone

Either 6 or 12 ml of flavanone synthase solution H were mixed with 10 µl of a solution of 12 or 24 µg 4,2′,4′,6′-tetrahydroxychalcone in ethyleneglycol monomethyl ether and 100 µl of an aqueous solution of 10 nmol *p*-coumaroyl-CoA and 24.4 nmol [2-^{14}C]-malonyl-CoA (1.1×10^6 dis./min), and incubated at 30 °C for 0.5 – 6 min (see Table 2). The reaction was stopped with 1 ml acetic acid, and the mixture was extracted four times with 20 ml diethyl ether. The ether was then evaporated, and the residue was dissolved in 0.5 ml methanol and chromatographed on Schleicher & Schüll No. 2043 paper in 30% acetic acid. The spots corresponding to naringenin, bis-noryangonin, and the chalcone were detected under ultraviolet light of 350 nm, cut out, and the radioactivity was measured as described above.

Protein Determination

Protein was measured by the biuret method (crude preparations) or by the direct spectrophotometric method at 260 and 280 nm [16].

Table 1. *Partial purification of flavanone synthase*
The relative amounts of naringenin and bis-noryangonin formed in the standard assay changed with increasing purity of the enzyme (for details see [7]). Specific activity and recovery of flavanone synthase beyond the $(NH_4)_2SO_4$ step were calculated from the sum of both products

Purification step	Protein	Specific activity	Purification	Recovery	Ratio of naringenin : bis-noryangonin formed
	mg	μkat/kg	-fold	%	
Dowex 1X2 supernatant	901	0.3	1	100	1:0
$(NH_4)_2SO_4$ fractionation, Sephadex G-25	102	1.6	5.4	69	1:0
DEAE-cellulose	14	15	50	62	1:0.33
Hydroxyapatite	1.1	92	310	30	1:0.43

RESULTS

Purification Procedure

A procedure resulting in about a 300-fold purification of the flavanone synthase with an overall yield of approx. 30% is summarized in Table 1. The flavanone synthase activity was separated on a DEAE-cellulose column from two other enzymes of the flavonoid glycoside pathway, chalcone isomerase and malonyl-CoA : flavonoid glycoside malonyl transferase (Fig. 1). In particular, a complete separation of the flavanone synthase and chalcone isomerase activities was achieved.

In addition to naringenin, increasing amounts of bis-noryangonin were obtained in the enzyme assay as the purity of the flavanone synthase increased beyond the $(NH_4)_2SO_4$ step. The formation of both products was always exclusively associated with the same protein fractions throughout the purification procedure. After the hydroxyapatite step, the flavanone synthase preparation was further fractionated by analytical disc gel electrophoresis. The enzyme activity catalyzing the formation of both naringenin and bis-noryangonin was again located in one sharp band [7]. Staining of the proteins on a separate gel revealed that the purification through the hydroxyapatite step had not yielded a homogeneous protein. Less than one half of the proteins visualized after staining of the gel with Coomassie brilliant blue R250 was associated with the synthase activity.

Stability of the Enzyme Activity

The flavanone synthase activity was very unstable at any stage throughout the purification when stored at 4 °C. Enzyme preparations which were purified only through the $(NH_4)_2SO_4$ step were stable at − 20 °C at least for several weeks after an initial loss of about one third of the activity upon freezing. More extensively purified enzyme preparations were highly unstable either at 4 °C or at − 20 °C. All attempts to stabilize the enzyme activity by the addition of polyalcohols, thiol reagents *etc.* have been unsuccessful.

Linearity of the Reaction with Time and Protein Concentration

The flavanone synthase reaction was linear with time over a period of at least 20 min at protein concentrations of up to 30 − 150 μg, depending upon the degree of purity, in the standard enzyme assay.

Dependence on pH and Temperature

The highest rate of product formation was found at about pH 8.0. The temperature optimum was at about 30 °C. Raising or lowering the temperature by 10 °C caused a decrease in the enzyme activity of about 25%.

Dependence on Ionic Strength

The synthase activity decreased considerably, when the ionic strength of the standard incubation mixture was decreased below 0.1 M potassium phosphate. Highest enzyme activity was obtained at about 0.1 − 0.3 M potassium phosphate. In the presence of, *e.g.*, 4 mM mercaptoethanol, the rate of naringenin formation changed even more drastically with variations in the ionic strength, but was also associated with relatively high rates of bis-noryangonin formation [7]. When Tris-HCl instead of potassium phosphate buffer was used, the rate of naringenin formation was reduced to about 50 − 60%.

Dependence on Substrate Concentrations

In accordance with the theoretical molar ratio of 3:1 for the formation of naringenin and 2:1 for the

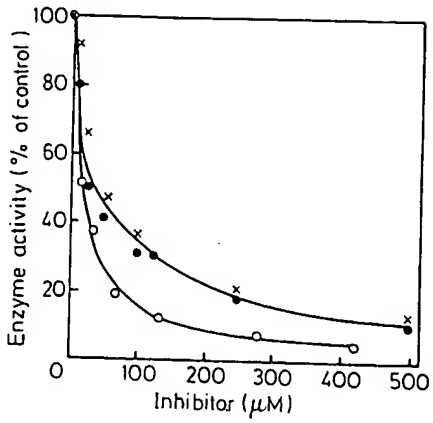

Fig. 2. *Inhibition of flavanone synthase activity by naringenin (○), CoASH (●), or pantetheine (×).* A purified enzyme preparation (solution H) was used. Naringenin, dissolved in 5 µl ethyleneglycol monomethyl ether, or CoASH or pantetheine, dissolved in 5 µl water, were added to the standard assay at the concentrations indicated

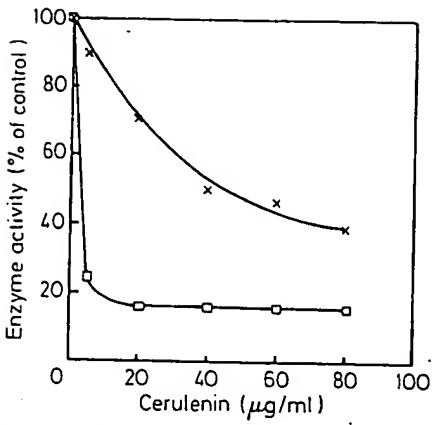

Fig. 3. *Inhibition of flavanone synthase activity by cerulenin.* Various amounts of the antibiotic dissolved in 5 µl ethanol were added to the standard assay either 10 min (□) or immediately (×) before the reaction was started by addition of the substrates. A purified enzyme preparation (solution H) was used.

formation of bis-noryangonin, the rates of formation of both products were proportional to time and protein concentration at 22 µM [2-^{14}C]malonyl-CoA and 9 µM *p*-coumaroyl-CoA (2.44 and 1 nmol per assay, respectively). No attempt was made to increase the substrate concentrations in the standard synthase asay above these values, due to the high costs of both CoA thiol esters.

Inhibition by Acetyl-CoA

Neither of the two products of the synthase reaction, naringenin or bis-noryangonin, was formed when acetyl-CoA replaced malonyl-CoA in the standard assay, using an enzyme preparation which had been purified through the DEAE-cellulose step. When acetyl-CoA was added to the complete synthase assay, concentrations varying from 0.13 to 2 µM were increasingly inhibitory. In the presence of 2 µM acetyl-CoA, the formation of naringenin was reduced to 50% of the control value.

Product Inhibition

Both naringenin and CoASH were potent inhibitors of the flavanone synthase reaction. When either product was added individually at a concentration of 10 µM to the standard enzyme assay, the synthesis of naringenin was inhibited by approx. 50%. Furthermore, a similar degree of inhibition was observed, when pantetheine replaced CoASH as an inhibitor. Fig. 2 shows further details of the dependence of the inhibitory effects of naringenin, CoASH, and pantetheine, respectively, on their concentrations in the enzyme assay.

Effects similar to those of naringenin were also obtained with the same molar concentrations of the isomeric chalcone. However, because of the rapid, non-enzymic isomerization of the chalcone to the flavanone under the conditions of the standard enzyme assay, the inhibition could have been due either partly or exclusively to the latter compound.

Inhibition by Cerulenin

An especially potent inhibitor was the antibiotic cerulenin. Fig. 3 shows the dependence of the degree of inhibition on the concentration of the antibiotic in the standard enzyme assay either with or without a preincubation period of 10 min. Under the conditions of preincubation, an inhibition of about 80% of the flavanone synthase reaction was achieved with 0.5 µg cerulenin (5 µg/ml).

Inhibition by Sulfhydryl Reagents

The enzyme activity was greatly inhibited by *p*-chloromercuribenzoate, *N*-ethylmaleimide, and iodoacetamide. An inhibition of about 50% was brought about by the addition of 1 µM *p*-chloromercuribenzoate, or 10 µM *N*-ethylmaleimide or iodoacetamide to the standard enzyme assay.

Effects of Divalent Cations and EDTA

Mg^{2+}, Ca^{2+}, Fe^{2+}, or EDTA, when added separately at 0.7 µM concentrations to the standard incubation mixture, had no significant effect on the synthase activity.

Table 2. *Relative amounts of products formed in the presence of chalcone*
Details of the experimental procedure are described under Materials and Methods. n.d. = not detectable above background radioactivity on chromatograms

Assay volume	Incubation time	Amount of chalcone added	10^{-3} × Radioactivity in		
			naringenin	bis-noryangonin	chalcone
ml	min	µg	dis./min		
6.1	6	12	210	115	n.d.
6.1	4	12	162	98	n.d.
6.1	2	12	125	63	n.d.
6.1	2	24	29	28	n.d.
12.1	0.5	24	65[a]	—[b]	n.d.

[a] Isolated by thin-layer chromatography on cellulose in 30% acetic acid and rechromatography in 15% ethanol. The radioactivity of the spot corresponding to naringenin was determined by scintillation spectrometry as described for spots from paper chromatograms under Materials and Methods.
[b] Not measured.

Identification of the Reaction Products

Of the two isomeric forms, the flavanone and the chalcone, only the flavanone was isolated as a radioactive product from the incubation mixture under the various conditions listed in Table 2. The assays were carried out for short periods of time, varying from 0.5 to 6 min, in the presence or absence of the chalcone, and the products were separated by chromatography. Large incubation volumes were used in these experiments in order to keep the chalcone at concentrations which inhibited the enzyme only moderately (see above) but provided amounts large enough to allow the reisolation and subsequent chromatographic detection of the chalcone, despite its rapid non-enzymic isomerization. While a considerably large portion of the total radioactivity was associated with the spots corresponding to naringenin and bis-noryangonin, the spots corresponding to the chalcone were in all cases free of radioactivity, except for the background level on the chromatograms. The lowest amount of label detectable in the chalcone spot within the limits of the experimental error would have been about 300 counts/min, or of the order of 0.1 to 1% of the radioactivity isolated as naringenin.

An enzyme preparation purified through the hydroxyapatite step was used for the experiments shown in Table 2. Under these conditions, about 30% of the radioactivity in the products of the synthase reaction were associated with bis-noryangonin. The structural identification of both products, naringenin and bis-noryangonin, is described elsewhere [1, 7].

Malonyl Transfer Reaction

The partially purified flavanone synthase obtained after the hydroxyapatite step catalyzed an efficient transfer of malonate from malonyl-CoA to panthetheine. The disappearance of malonyl-CoA concomitant with the formation of malonyl pantetheine in the presence of an excess of panthetheine in the standard enzyme assay is shown in Fig. 4. The malonyl pantetheine formation increased at a linear rate with increasing amounts of protein at least in the range from 1 to 16 µg per assay. The sum of both malonyl thiol esters remained constant under these conditions

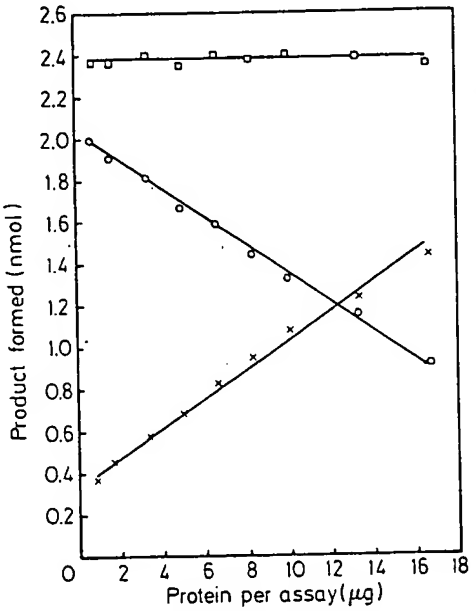

Fig. 4. *Formation of [2-^{14}C]malonyl pantetheine (×) from [2-^{14}C]-malonyl-CoA (○) and pantetheine in the presence of various amounts of flavanone synthase.* A purified enzyme preparation (solution H) was used for the malonyl transfer reaction as described under Materials and Methods. No detectable amount of malonyl pantetheine was formed, when a heat-denatured enzyme solution was used. (□) Sum of both malonyl thiol esters

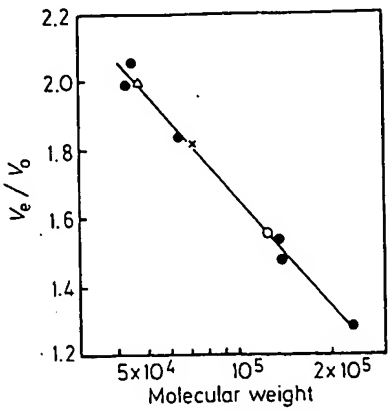

Fig. 5. *Estimation of the molecular weights of flavanone synthase (○), malonyl-CoA : flavonoid glycoside malonyl transferase (×) and chalcone isomerase (△).* A flavanone synthase preparation (solution S) containing all the three enzyme activities was used for column chromatography on Sephadex G-200 with buffer A. The column was equilibrated (●) according to the method of Andrews [37] with blue dextran, catalase, alcohol dehydrogenase, hemoglobin, the monomeric and dimeric forms of bovine serum albumin, and ovalbumin. V_e = elution volume, V_o = void volume

suggesting that an enzyme-mediated hydrolysis of either product had not taken place during the incubation. In contrast to the overall flavanone synthase reaction, the malonyl transfer reaction was not inhibited by naringenin at the concentrations which were used for the experiments shown in Fig. 2.

Molecular Weights of Flavanone Synthase and Two Metabolically Related Enzymes

The apparent molecular weights of flavanone synthase, chalcone isomerase, and malonyl-CoA : flavonoid glycoside malonyl transferase, all of which were isolated together after the $(NH_4)_2SO_4$ step, were estimated from the elution volumes from a calibrated Sephadex G-200 column. Fig. 5 shows that values of about 120000, 50000, and 70000, respectively, were obtained for the three enzymes, with an experimental error of about ± 10%.

DISCUSSION

Light-induced cell suspension cultures of *Petroselinum hortense* have been extensively used in recent years to investigate the mechanisms of flavonoid glycoside formation at an enzymic level [1, 13, 15, 17–25]. In the course of these studies we proposed a hypothetical scheme for this pathway which included the possibility of a chalcone being the first intermediate in the sequence of reactions [18, 26]. Accordingly, a chalcone synthase was originally postulated to catalyze the formation of this compound from *p*-coumaroyl-CoA and malonyl-CoA [6, 18, 27]. Our present results demonstrate, in contrast to this hypothesis, that the flavanone is the immediate product of the synthase reaction. The function *in vivo* of the chalcone isomerase appears therefore to be the formation of the chalcone from the flavanone, rather than the reverse reaction. This would be in agreement with the recently proposed isomerization of naringenin by chalcone isomerase in soybean [28].

Several properties of the flavanone synthase suggest that the reaction mechanism closely resembles that of the condensation steps involved in the syntheses of fatty acids [29] and 6-methylsalicylic acid [30]. In all of these cases, the acyl moieties of either acetyl-CoA (fatty acid and 6-methylsalicylic acid syntheses) or *p*-coumaroyl-CoA (naringenin and bis-noryangonin syntheses) function as primers in subsequent condensation reactions with malonyl-CoA, resulting in a stepwise elongation of the carbon chain by acetate units. Various sulfhydryl reagents, which in the case of fatty acid synthesis were shown to inhibit specific reactions involved in the condensation steps, were also potent inhibitors of 6-methylsalicylic acid and flavanone synthesis.

Another line of evidence for the close similarity between the mechanisms of chain elongation in fatty acid and flavanone syntheses was the inhibitory effect of cerulenin. This antibiotic inhibits specifically the β-ketoacyl–acyl-carrier-protein synthetase reaction [31, 32], a central step in fatty acid synthesis, as well as the formation of leucomycin, which is also derived from acetate units [33].

In further analogy to fatty acid synthesis [34], the partially purified flavanone synthase efficiently catalyzed the transfer of the malonyl residue from malonyl-CoA to pantetheine. It cannot be deduced from the present data, whether this reaction represents (a) merely the reversal of a transfer of malonate from malonyl-CoA to the enzyme, or (b) the transfer of enzyme-bound malonate to a second site which might be equivalent to the pantetheinyl residue of the fatty acid synthetase complex from yeast [35, 36]. In either case, pantetheine would serve as an artificial analogue of the natural carrier of the malonyl residue. Thus the malonyl transfer reaction might be one explanation for the strong inhibitory effects of pantetheine and CoASH on the flavanone synthase activity.

A scheme for the sequence of reactions involved in the enzyme-mediated formation of naringenin and bis-noryangonin is proposed in Fig. 6. The molecular weight of about 120000 determined for the flavanone synthase appears to be sufficiently large to assume that this enzyme catalyzes the different individual steps which are postulated. Since no reduction

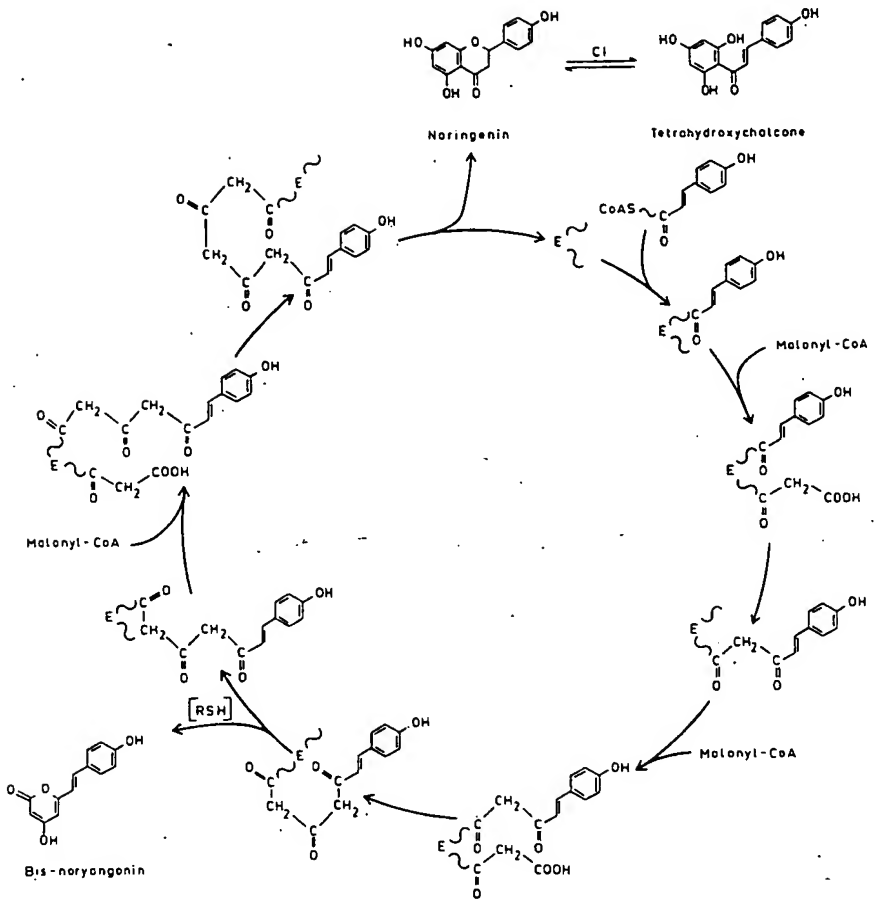

Fig. 6. *Hypothetical scheme for the formation of naringenin and bis-noryangonin from* p-*coumaroyl-CoA and malonyl-CoA*. Details of the conditions for bis-noryangonin formation are described elsewhere [7]. E = enzyme (flavanone synthase), RSH = mercaptoethanol or dithioerythritol. The scheme includes the isomerization of naringenin to the corresponding chalcone catalyzed by chalcone isomerase (CI). The waved lines represent energy-rich bonds, probably thiol esters

of an intermediate is involved in the proposed mechanism, the formation of naringenin probably requires fewer steps than the formation of fatty acids or of 6-methylsalicylic acid. It seems likely, however, that the synthesis of those naturally occurring flavonoids which lack the hydroxyl group in the 5-position includes a reduction step of an enzyme-bound intermediate similar to that involved in 6-methylsalicylic acid synthesis [30].

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